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ALKALINE PHOSPHATASE FROM *BACILLUS LICHENIFORMIS*

SOLUBILITY DEPENDENT ON MAGNESIUM, PURIFICATION AND CHARACTERIZATION

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Summary

The membrane-associated alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) from *Bacillus licheniformis* MC14, a facultative thermophile, was purified to homogeneity in buffer containing 0.2 M Mg^{2+}. The alkaline phosphatase purified in this manner is insoluble upon removal of the magnesium by dialysis. This insoluble alkaline phosphatase has been characterized and compared to the previously purified heat-solubilized enzyme (Hulett-Cowling, F.M. and Campbell, L.L. (1971) Biochemistry 10, 1364—1371).

Introduction

Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) has been found in a number of organisms, both procaryotic and eucaryotic [1—6].

Escherichia coli alkaline phosphatase is a soluble protein which is located in the periplasmic space; thus a crude enzyme preparation can be obtained upon formation of protoplasts. The alkaline phosphatase from *Bacillus subtilis* [4] or from *Bacillus licheniformis* [1] is not soluble in aqueous buffer and will pellet when centrifuged at 100 000 × *g* for 60 min. The alkaline phosphatase from both organisms can be extracted from the membrane fraction of lysed cells with buffer containing 1 M Mg^{2+} and will remain soluble in magnesium concentrations as low as 0.2 M [7].

The membrane-associated alkaline phosphatase from *B. licheniformis* MC14, a facultative thermophile, can be rendered soluble in aqueous buffer without magnesium by the following treatment: heating to 80°C for 5 min in the presence of 1 M Mg^{2+}. When Mg^{2+} is removed by dialysis, the alkaline phospha-

tase remains soluble upon centrifugation at 100 000 × *g* for 1 h. If the Mg^{2+}-extracted enzyme is not subjected to this drastic heat step, it will precipitate upon removal of the magnesium [1]. The alkaline phosphatase from *B. subtilis* has not been heat solubilized and depends on Mg^{2+} concentrations of at least 0.2 M for solubility [4]. It has been purified and characterized in the presence of Mg^{2+}. A similar approach was used to purify the alkaline phosphatase from *B. licheniformis*. We felt that studies to determine how heating alters the enzymes during the drastic heat step to confer solubility could be approached first by characterizing the enzyme which had not undergone the heat step.

In order to make direct comparisons with the salt-heat-purified enzyme we repeated a number of the characterizations of this enzyme in the presence of magnesium at concentrations equal to that in the salt-dependent alkaline phosphatase assays. The addition of Mg^{2+} was essential since removal of the Mg^{2+} causes the insoluble salt-dependent alkaline phosphatase enzyme purified in the presence of 0.2 M Mg^{2+} to precipitate, which would be an unsuitable preparation for performing duplicate assays. Any differences from the values previously reported for the salt-heat enzyme [1,5] are noted in this paper.

Purifying the membrane-associated alkaline phosphatase from *B. licheniformis* without the heat step was also desirable in that it yielded an enzyme which is more like the alkaline phosphatase on the membrane and therefore would be a better source of enzyme for reconstitution studies with enzyme-depleted membranes and to determine whether there are specific sites for binding of the alkaline phosphatase to the bacterial membrane. Histochemical localizations have shown discrete sites of enzymatic activity on the inner surface of the plasma membrane [8]. Our recent discovery [7] of a soluble alkaline phosphatase in *B. licheniformis*, which can be released upon spheroplast formation, makes the characterization of the particular enzyme more valuable if further studies show that the particulate enzyme is a precursor of the soluble alkaline phosphatase. Preliminary data indicates that this may be the case since the amount of soluble alkaline phosphatase increases as the amount of membrane-associated alkaline phosphatase decreases during growth [7].

Materials and Methods

The organism used in this study is a facultatively thermophilic strain of *B. licheniformis* MC14 [1]. Stocks were maintained on slants of 2% tryptone (Difco) and 2% agar (Difco) (pH 7.2).

For inoculation into liquid media cells were incubated overnight (approx. 14 h) on tryptone-agar plates. The inoculum for fermentor growth was grown in four 1000-ml flasks containing 400 ml 1.5% neopeptone broth and 0.1% fructose on a water bath shaker at 55° C until the absorbance at 540 nm was 0.6—0.8 (10 mm path length). At this time, the 1600 ml culture was used to inoculate a 14 l New Brunswick fermentor which contained 12 l 1.5% neopeptone and 0.1% fructose (agitation at 400 rev./min and aeration of 10 l/min at 55° C). Cells were harvested at peak enzyme production, usually at an absorbance (540 nm) of 1.2—1.4, by centrifugation in a Sharples centrifuge and frozen until used.

Assays. Alkaline phosphatase was assayed by adding enzyme to *p*-nitrophe-

nyl phosphate (1.0 mM in 1.0 M Tris/acetate, pH 8.0) at 55° C. The reaction was stopped by the addition of 0.5 ml 13% K_2HPO_4 to 2.5 ml assay mixture. For all of the assays except those performed in the purification, the initial velocity of the hydrolysis of *p*-nitrophenyl phosphate was determined by the use of a Gilford recording spectrophotometer equipped with a constant temperature cuvette chamber.

Inorganic phosphate was determined by the method of Fiske and SubbaRow [9]. Succinate dehydrogenase, NADH-cytochrome *c* reductase, and NADH-oxidase were assayed by the method of Reaveley and Rogers [10].

Sodium dodecyl sulfate (SDS) gels. Electrophoresis through polyacrylamide gels in the presence of SDS was done according to the procedure of Fairbanks et al. [11].

Substrate specificity. Substrates (*p*-nitrophenyl phosphate, 5'-AMP, 3'-AMP, 5'-CMP, 5'-IMP, 5'-UMP, 5'-ATP, 3-phosphoglyceric acid, D-glucose 6-phosphate, β-glycerolphosphate, pyrophosphate, and *p*-nitrophenyl sulfate) were all purchased from Sigma. Substrate solutions were made up to concentrations of $1.0 \cdot 10^{-2}$ M in 1.0 M Tris/acetate buffer (pH 8.0). Assays for hydrolysis were performed by incubating pure salt-dependent alkaline phosphatase and pure heat-solubilized alkaline phosphatase with 2 ml substrate and allowing hydrolysis to proceed for 1, 3 and 5 min. The reaction was stopped by the addition of 1 ml 20% trichloroacetic acid. If a precipitate formed, the assay mixture was centrifuged at top speed in a clinical centrifuge for 10 min. Aliquots of the resulting supernatant fraction (0.5 and 1.0 ml) were then added to water (deionized) to give a final volume of 3 ml. Control blanks were obtained by adding the trichloroacetic acid to the substrate before adding the enzyme.

Amino acid analysis. Amino acid analysis was performed on a Beckman automatic amino acid analyzer. Duplicate samples of enzyme (120 μg/ml) were hydrolyzed in 6 M HCl for 24, 48 and 72 h. Aliquots of enzyme which would yield a protein concentration of 0.12 mg/ml when suspended in the carrier buffer were lyophilized to dryness. 1 ml of 6 M HCl was added to each ampoule and the ampoule was sealed under vacuum. The sealed ampoules were placed in an oven at 110° C for various times. The ampoules were then opened and the HCl drawn off under vacuum. Tryptophan was measured spectrophotometrically by the method of Edelhoch [12].

Molecular weight determination. Native molecular weight was determined by the method of Yphantis [13]. A Beckman model E ultracentrifuge was used with the AND rotor and a six sector Yphantis cell. Protein concentrations of 0.92, 0.72 and 0.52 mg/ml were placed in each of three of the sectors. Buffer containing no protein was placed in each of the other sectors as reference. The buffer was 0.01 M Tris/acetate (pH 7.3)/0.1 mM $CoCl_2$/0.2 M magnesium acetate.

Temperature stability. To determine temperature stability purified salt-dependent alkaline phosphatase and purified salt-heat alkaline phosphatase were incubated in 0.01 M Tris/acetate buffer (pH 7.3)/0.1 mM $CoCl_2$/1.0 mM magnesium acetate at 70° C for various times. The zero time value was obtained by assaying as soon as the enzyme reached 70° C. The assay was performed as described above.

Results

Association of alkaline phosphatase with the membranes

In order to show that the alkaline phosphatase is associated with the cytoplasmic membrane, before extraction with magnesium salt, isopycnic sucrose density gradients (30—55%) were run. Cells in 0.1 M Tris/acetate (pH 7.3)/0.1 mM $CoCl_2$ containing 20% sucrose were converted to protoplasts with lysozyme (1 mg/ml) and then harvested by low speed centrifugation (2500 × *g*, 20 min). The harvested protoplasts were then suspended in buffer without sucrose and allowed to lyse gently. The lysed membrane fraction was centrifuged to separate it from soluble cellular components, suspended in buffer, and layered onto 30—55% isopycnic sucrose gradients. These were run at 148 000 × *g* for 18 h. The tubes were punctured, 0.5-ml fractions were collected and assayed for alkaline phosphatase, succinate dehydrogenase, NADH-cytochrome *c* reductase, NADH oxidase and for protein (280 nm absorbing material). The enzymes were assayed as outlined by Reaveley and Rogers [10]. Fig. 1 shows two peaks of material absorbing at 280 nm within the gradient and the alkaline phosphatase activity is associated with the peaks. Reaveley and Rogers [10] have previously shown that the cytoplasmic membrane of *B. licheniformis* fractionates into two 280-nm absorbing components of different densities when separated on isopycnic sucrose gradients (30—55% sucrose). They also found dense material at the bottom of the gradient and established that these components were membrane material by assaying for specific enzymes (succinate dehydrogenase, NADH-cytochrome *c* reductase, and NADH oxidase) known to be associated with the membrane in *B. licheniformis*. The distribution of these enzyme membrane markers was analogous in our experiment to that shown by Reaveley and Rogers [10].

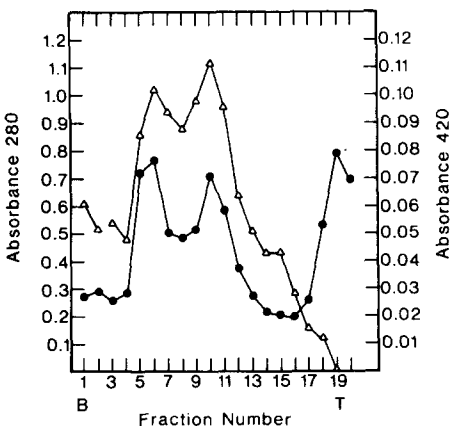

Fig. 1. Sucrose density gradient centrifugation of washed membranes. Consecutive 0.5-ml fractions were collected and analyzed for protein (A_{280nm}) and enzyme activity (A_{420nm}). The procedure for the membrane preparation and enzyme assays to confirm membrane isolation are given in Materials and Methods. As the figure shows alkaline phosphatase is associated with the membrane fractions. ●———●, A_{280nm}; △———△, A_{420nm}.

Purification of the enzyme

One major objective of this study was to purify the membrane-associated alkaline phosphatase without solubilizing it via the drastic heat step used previously [1]. The alkaline phosphatase from *B. subtilis* has been previously purified and characterized in its insoluble form [4]. A similar purification scheme was used here.

Frozen fermentor grown cells (typically 50—100 g wet weight) were lysed by incubation with 1.0 mg/ml lysozyme and 0.05 mg/ml DNAase at 37°C overnight with gentle shaking. This crude lysate was then centrifuged at 40 000 × *g* for 60 min. More than 80% of the alkaline phosphatase activity was found in the pellet. The pellet fraction was suspended in buffer and centrifuged at 40 000 × *g* for 1 h. The pellet was then suspended in buffer containing 1 M Mg^{2+} and stirred for 2 h at 0°C. This suspension was then centrifuged at 40 000 × *g* for 60 min. The alkaline phosphatase activity was in the supernatant fraction.

The magnesium salt was removed from the alkaline phosphatase-containing supernatant fraction by dialysis. An amorphous precipitate formed which contained the alkaline phosphatase activity. It was centrifuged at 20 000 × *g* for 30 min and suspended in 0.01 M Tris/acetate buffer (pH 7.3)/0.1 mM $CoCl_2$/0.5 M Mg^{2+}. This was stirred for 2 h at 0°C and centrifuged at 40 000 × *g* for 60 min. The supernatant fraction, which contained the alkaline phosphatase activity, was again dialyzed to remove Mg^{2+}. The precipitated enzyme was collected by centrifugation as above and the pellet fraction was extracted with 0.2 M magnesium acetate. The suspension was centrifuged at 40 000 × *g* for 30 min and the supernatant fraction which contained the enzyme activity was concentrated by pressure dialysis using a Diaflow PM-10 membrane. The concentrated enzyme was then layered onto the first in a series of three 5—20% sucrose gradients.

Fig. 2 shows the protein and enzyme activity recovered from the first and

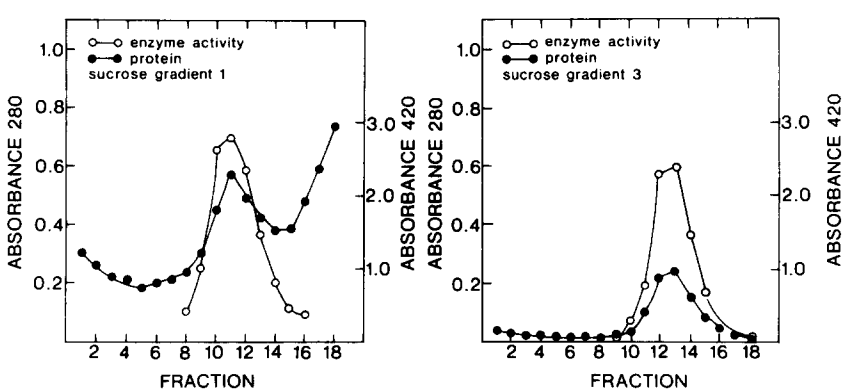

Fig. 2. First and third sucrose gradients in purification of salt-dependent alkaline phosphatase. Concentrated enzyme soluble in buffer with 0.2 M Mg^{2+} was layered onto a 5—20% linear sucrose gradient (gradient 1) which was centrifuged at 35 000 rev./min in a Beckman SW-41 rotor for 24 h. 0.5-ml fractions were collected and the peak enzyme fractions were pooled, dialyzed to remove sucrose, concentrated and layered onto the second sucrose gradient. After the third sucrose gradient the alkaline phosphatase profile reflects the protein profile. Symbols: ○———○, enzyme activity; ●———● protein.

third sucrose gradients. As the figure shows, the major protein component layered onto the first gradient is alkaline phosphatase and the third gradient is homogeneous alkaline phosphatase. Fig. 3 shows a single protein band when the alkaline phosphatase was analyzed on SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue. Table I shows the steps in the purification, the specific activities and the degree of purification at each step. A 1000-fold purification was typical.

Determination of native molecular weight

The molecular weight of the purified alkaline phosphatase was determined by the sedimentation equilibrium method of Yphantis [13]. The graph of $\log [Y_r - Y_0]$ vs. r^2 where r is equal to the distance from the center of rotation, Y_r is the y value (vertical fringe displacement) at any r and Y_0 is the value of y at the meniscus yields a straight line which suggests homogeneity of the sample. The slope was used to calculate the molecular weight, which is 124 000 ± 3000. The density of the solvent was determined pycnometrically and the partial specific volume was determined from the amino acid composition. The molecular weight is 124 000 ± 3000 for the salt-dependent alkaline phosphatase compared to 121 000 ± 3000 for the salt-heat alkaline phosphatase [5].

Subunit molecular weight on SDS gels

The subunit molecular weight of salt-dependent alkaline phosphatase and salt-heat alkaline phosphatase was determined on SDS gels using the method of Fairbanks et al. [11]. The subunit molecular weight of the salt-dependent alkaline phosphatase is approx. 60 000 as compared to the approximate subunit molecular weight of 55 000 for the salt-heat alkaline phosphatase. Although the difference seen here is small and possibly insignificant, a number of trials consistently indicated a molecular weight of 60 000 ± 3000 for salt-dependent alkaline phosphatase and 58 000 ± 3000 for the salt-heat alkaline phosphatase.

Amino acid composition

The amino acid composition of the pure salt-dependent alkaline phosphatase was determined by hydrolysis of the pure enzyme with constant boiling HCl in a sealed ampoule under reduced pressure for various times (24, 48 and 72 h). The results are shown in Table II. The amino acid composition of the salt-heat alkaline phosphatase has been previously determined [1]. A high lysine content in addition to high glutamate/glutamine and aspartate/asparagine were found in both enzyme preparations. The two forms of the enzyme are very similar for

Fig. 3. SDS-polyacrylamide gel showing the pooled peak fractions after the third sucrose gradient (see Fig. 1). The gel system used was that of Fairbanks et al. [11] which utilized a 5.6% acrylamide gel. The wire represents the Pyronin-y tracking dye.

TABLE I

PURIFICATION OF SALT-DEPENDENT ALKALINE PHOSPHATASE FROM *B. LICHENIFORMIS*

Fraction	Volume	Protein (mg)	Units	Specific activity	Purification (-fold)
Crude lysate	77.0	26 796.0	5082.0	0.2	—
Crude lysate pellet	59.0	26 196.0	6619.8	0.3	1.3
1 M Mg^{2+} extract	50.5	974.9	2555.3	2.6	13.8
Pellet from dialysis	34.8	415.1	1033.6	2.5	13.1
0.5 M Mg^{2+} extract	34.6	286.8	2321.7	8.1	42.6
Pellet from dialysis	32.0	160.2	1971.2	12.3	64.7
0.2 M Mg^{2+} extract	31.0	62.57	1016.2	16.24	81.2
Pooled fractions sucrose gradient 1	11.2	5.2	1084.0	209.7	1103.7
Pooled fractions sucrose gradient 2	5.8	1.2	287.1	247.5	1302.6
Pooled fractions sucrose gradient 3	3.2	0.6	110.9	198.0	1042.1

most of the residues; however, arginine, aspartic acid or asparagine, glycine, tryptophan and especially proline, show differences. Proline was not detected in any of the 12 samples of salt-dependent alkaline phosphatase hydrolyzed and analyzed, whereas proline was found in all 18 samples analyzed of the salt-heat alkaline phosphatase. The explanation for this has not been found as yet.

TABLE II

AMINO ACID COMPOSITION OF *B. LICHENIFORMIS* SALT-DEPENDENT ALKALINE PHOSPHATASE

Amino acid	Time of hydrolysis (h)				Salt-heat alkaline phosphatase
	24	48	72	Average [a]	
Lysine	124	130	108	132	127
Histidine	20	22	38	21	17
Arginine	14	10	14	13	33
Aspartic acid or asparagine	106	96	96	99	130
Threonine [b]	60	60	62	61	68
Serine [b]	66	64	72	67	79
Glutamic acid or glutamine	100	110	94	101	107
Glycine	86	86	76	86	108
Alanine	92	94	74	93	104
Valine [c]	58	62	78	78	105
Methionine [b]	36	34	38	36	32
Isoleucine	40	44	42	42	48
Leucine	56	60	60	59	70
Tyrosine	58	54	62	58	32
Phenylalanine	32	28	30	30	33
Tryptophan [d]	16	16	18	17	8
1/2-cystine	0	0	0	0	0
Proline	0	0	0	0	44

[a] Values averaged where no evidence of destruction or further release with time was noted, i.e. lysine based on 24- and 48-h values.

[b] Values extrapolated to zero time.

[c] 72 h hydrolysis values used.

[d] Determined by the method of Edelhoch [12].

pH optimum

The pH optimum of salt-dependent alkaline phosphatase and salt-heat alkaline phosphatase was determined by adjusting 0.05 M triethanolamine · HCl buffer to the appropriate pH. The buffer contained 1 mM *p*-nitrophenyl phosphate as substrate. The substrate (2 ml) was brought to 55°C and 10 µl enzyme (salt-dependent or salt-heat alkaline phosphate, both in buffer containing 0.2 M Mg^{2+}) was added and the reaction rate monitored. The pH was checked immediately before the experiment was performed. The amounts of enzyme used were 0.12 mg/assay for the salt-dependent alkaline phosphatase and 0.26 mg/assay for the salt-heat alkaline phosphatase. The results show the same pH optimum, 10.1, for both the salt-dependent and salt-heat alkaline phosphatase. However, the salt-dependent alkaline phosphatase is active over a greater range as shown by its high activity at pH 11.2 whereas the salt-heat alkaline phosphatase shows a sharp drop in activity at pH 11.0.

Temperature stability

To determine temperature stability purified salt-dependent alkaline phosphatase and purified salt-heat alkaline phosphatase were incubated in 0.01 M Tris/acetate buffer (pH 7.3)/0.1 mM $CoCl_2$/1 mM magnesium acetate at 70°C for various times. The zero time value was obtained by assaying the enzyme as soon as the enzyme reached 70°C. Salt-heat alkaline phosphatase activity falls off rapidly in the first 10 min but then levels off and retains about 42% activity through 30 min. The salt-dependent enzyme inactivated more slowly but after 15 min the rate of inactivation is greater than for salt-heat enzyme. The amount of inactivation during the first 5 min is virtually identical for the salt-heat and the salt-dependent alkaline phosphatase.

K_m determination

The K_m of salt-dependent alkaline phosphatase was determined for *p*-nitrophenyl phosphate by incubating enzyme (5.8 µg/assay) with various concentrations of substrate ($1.0 \cdot 10^{-5}$—$1.0 \cdot 10^{-3}$ M). The substrate (2 ml) was brought to 55°C and then added to each of three cuvettes in a Gilford 2400 recording spectrophotometer equipped with a thermostated cuvette chamber. The enzyme was added with rapid stirring to cuvettes 2 and 3 while cuvette 1 served as the control. The velocity measured $\frac{\Delta 420nm}{\Delta t}$ was converted to units of activity and a Lineweaver-Burk double reciprocal plot was graphed. The apparent K_m for *p*-nitrophenyl phosphate was $2.52 \cdot 10^{-4}$ M.

Since there was no magnesium present in the buffer containing the salt-heat alkaline phosphate at the time the kinetics were determined [1], K_m and K_i were determined with salt-heat enzyme in buffer containing 0.2 M Mg^{2+} which is identical to the Mg^{2+} concentration in the buffer containing the salt-dependent enzyme. Accounting for the dilution into the substrate the final magnesium concentration in the assay was 1 mM. The amount of salt-heat enzyme added to each assay was 2.6 µg. The K_m determined in this manner for the salt-heat enzyme is $4.0 \cdot 10^{-4}$ M.

K_i determinations were performed by the method of Dixon [14]. The inhibitor used was inorganic phosphate (as K_2HPO_4) and was added to *p*-nitrophenyl phosphate ($1.1 \cdot 10^{-3}$ M) to yield final inhibitor concentrations ranging from

TABLE III

SUBSTRATE SPECIFICITY OF SALT-DEPENDENT ALKALINE PHOSPHATASE AND SALT-HEAT ALKALINE PHOSPHATASE RELATIVE ACTIVITY

Results are expressed in % *P*-nitrophenyl phosphate. n.r., no reaction.

Substrate	Salt-dependent alkaline phosphatase
P-Nitrophenyl phosphate	100.0
5'-AMP	86.8
3'-AMP	62.3
5'-CMP	77.4
5'-IMP	92.5
5'-UMP	81.1
5'-ATP	11.3
3-Phosphoglycerate	28.3
D-Glucose 6-phosphate	30.2
β-Glycero	35.9
PP_i	66.6
P-Nitrophenyl sulfate	n.r.

$1.0 \cdot 10^{-3}$ to 0.1 M. The temperature was kept at a constant 55°C when enzyme (5.8 μg/assay salt-dependent, and 2.6 μg/assay salt-heat alkaline phosphatase) was added to the cuvettes. Activity was determined as above (K_m determinations). The apparent K_i for inhibition by P_i is $1.1 \cdot 10^{-2}$ M for the salt-dependent alkaline phosphatase as contrasted to an apparent K_i of $2.5 \cdot 10^{-3}$ M for the salt-heat alkaline phosphatase.

Hydrolysis of substrates by salt-dependent alkaline phosphatase

The hydrolysis of various substrates by pure salt-dependent alkaline phosphatase is shown in Table III. The alkaline phosphatase from both purification schemes (salt-dependent and salt-heat alkaline phosphatase [1]) shows high rates of hydrolysis of the nucleotide monophosphates. Lower reactivity was shown toward ATP, β-glycerolphosphate, 3-phosphoglyceric acid and D-glucose 6-phosphate.

Discussion

Previous work utilizing electron microscopy has indicated that the alkaline phosphatase is membrane associated [8]. This was not confirmed biochemically, however, until the isolated membranes were fractionated on isopycnic sucrose gradients as we have reported here. This supports the electron microscopic evidence and makes the insolubility of the alkaline phosphatase consistent with those of many other membrane proteins which are also insoluble after extraction from the membrane.

While characterizing the salt-dependent alkaline phosphatase we find many similarities to the salt-heat enzyme which are to be expected considering the common origin of the enzyme. The molecular weights are virtually identical given the limitations of the methods used for this determination. This indicates that whatever changes have taken place during the heat step to solubilized the enzyme have not had a drastic effect on the molecular weight.

The amino acid composition is also quite similar which again is not unexpected. Other techniques such as limited proteolysis and peptide mapping coupled with amino acid analysis of certain peptides might help clarify any apparent differences observed in the amino acid analysis of two enzyme forms. These methods may be employed to help elucidate how heating confers solubility.

Some other properties which are similar include pH optimum, thermal stability, K_m and substrate utilization. The pH optimum of the salt-heat alkaline phosphatase is affected by the presence of magnesium in the assay system. The enzyme preparation used previously [1] did not contain magnesium and a pH optimum of 8.5 was reported. In a system with magnesium a pH optimum of 10.1, which is identical to the pH optimum of the salt-dependent enzyme, is seen. The activity of the two enzyme forms is different over the pH range examined with the salt-dependent alkaline phosphatase retaining a greater percent of the maximum activity at higher pH values. The heat step could have rendered the salt-heat enzyme more sensitive to pH changes by removing some factor or causing a slight conformational change.

Thermal stability is virtually identical between the salt-dependent and the salt-heat alkaline phosphatase over the first 5 min of incubation at 70° C. .

The K_m values of the two enzyme forms are also quite similar. The K_m of the salt-heat enzyme was previously determined to be $6.0 \cdot 10^{-4}$ M for *p*-nitrophenol phosphate in buffer with no magnesium present [1]. In this study we have determined the K_m of salt-heat alkaline phosphatase in the presence of magnesium to be $4.0 \cdot 10^{-4}$ M, an increase in substrate affinity of about 30%. This value is closer to the value determined for salt-dependent alkaline phosphatase (also in magnesium) of $2.5 \cdot 10^{-4}$ M.

Substrate utilization is another area of great similarity between the two enzyme forms. Both the salt-heat and salt-dependent show high phosphatase activity on nucleotide monophosphates. The previously determined substrate specificity for salt-heat [1] was not in the presence of magnesium. We have assayed the phosphatase activity of salt-heat in the presence of 1.0 mM Mg^{2+} using ATP and pyrophosphate as substrates. There was an increase in hydrolyzing activity (unpublished results) as compared to that reported by Hulett and Campbell [1]. Therefore it is possible that the activity of the salt-heat enzyme would be greater on the other substrates in the presence of magnesium as well.

When salt-dependent alkaline phosphatase is allowed to react with antisera prepared against salt-heat alkaline phosphatase on Ouchterlony double diffusion plates the precipitin lines indicate total immunological identity [15]. This further confirms the close relationship between the two enzyme forms.

The major differences between the two enzymes forms are solubility in aqueous buffer and K_I values in the presence of magnesium. The apparent K_I value for the salt-heat enzyme is $3.7 \cdot 10^{-2}$ M in aqueous buffer [1] but drops to $2.5 \cdot 10^{-3}$ M in buffer containing 1.0 mM magnesium. This increase in sensitivity to inorganic phosphate as inhibitor could be due to the presence of magnesium, either as an inhibitor itself or by causing the P_i to be more accessible to the active site of the enzyme. The former possibility is not likely, however, since the presence of magnesium enhanced the hydrolysis of ATP and PP_i by the salt-heat alkaline phosphate which suggests that the accessibility of P_i or

the binding constant of P_i to the active site is greater in the presence of magnesium. The apparent K_I of salt-dependent alkaline phosphatase, $1.1 \cdot 10^{-2}$ M, shows it is not as sensitive to inhibition by inorganic phosphate as the salt-heat enzyme.

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